IMMUNOLOGICAL INVESTIGATIONS OF RELATIONSHIPS WITHIN THE TEREBRATULID BRACHIOPODS

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ABSTRACT. Intra-crystalline macromolecules isolated from the skeletons of nine species of Recent articulate brachiopods were compared by enzyme linked immunosorbent assay (ELISA) to assess the relationships within the Order Terebratulida (Phylum Brachiopoda, Class Articulata). Immunological distance data indicated that the sub-division of this order into three suborders (based on the characteristics of the internal skeleton, particularly the brachial loop) is not valid. Three major clusters were recognized within the Terebratulida which approximately correspond to recognized superfamilies, with the exception that the family Kraussinidae (possessing a long brachial loop) is placed within the Terebratulacea (characterized by a short loop). The three lineages are more closely related to each other than was previously predicted, and yet within most lineages there is a greater degree of subsequent diversification than has hitherto been recognized. The independent evolution of long brachial loops in two lineages identified by the immunological data, highlights problems with using the internal skeleton as a high-level taxonomic character within the terebratellids.

THE Terebratulida is the largest extant order of brachiopods, being characterized by the possession of an internal skeleton (brachidium or brachial loop) which in life supports the lophophore. A taxonomic treatment incorporating patterns of loop development was first elaborated by Beecher (1893), extended by Muir-Wood (1955), and utilized in the Treatise (Williams *et al.* 1965) in which the Order Terebratulida is divided into three suborders on the basis of the ontogeny and form of loop development. Two suborders characterized as possessing either short- (Terebratulidina) or long-loops (Terebratellidina), contain extant members; the third (Centronellida) is extinct. The prevailing taxonomic situation of the extant families is summarized by Collins *et al.* (1988); although inadequacies within the systematics of the Terebratulida have been recognized (e.g. Richardson 1975; Elliott 1976; Williams and Hurst 1977) the essential sub-division has remained unchallenged (Text-fig. 1*a*).

In the earlier immunological study, we presented data which appeared to challenge the primacy of loop length as a subordinal character within the terebratulids (Collins *et al.* 1988). No immunological distances were illustrated, and the systematic conclusions were restricted, since only three sera were used to establish the relationships among the various extant genera. The implications that this earlier study had for the systematics of the brachiopods prompted us to conduct a detailed investigation using a more robust immunological distancing approach.

Immunological distance measurements have frequently been used in taxonomic investigations, either using whole organism extracts (Olsen-Stojkovich *et al.* 1986; Price *et al.* 1987) or purified macromolecules (Sarich and Wilson 1967; Lowenstein *et al.* 1981; Lowenstein 1985). The technique involves the preparation of antisera against each taxon studied and reciprocal determinations of all sera against all taxa. In interpreting immunological distance, the technique assumes that the rate of evolution averaged over a large number of antigenic sites is uniform enough to give an accurate portrayal of the evolutionary branching pattern of the groups examined; for serum proteins it has been demonstrated that immunological distance closely parallels protein evolution (e.g. Maxon and Maxon 1979). In addition, within the cancellothyrid genus *Terebratulina* (which is used in this

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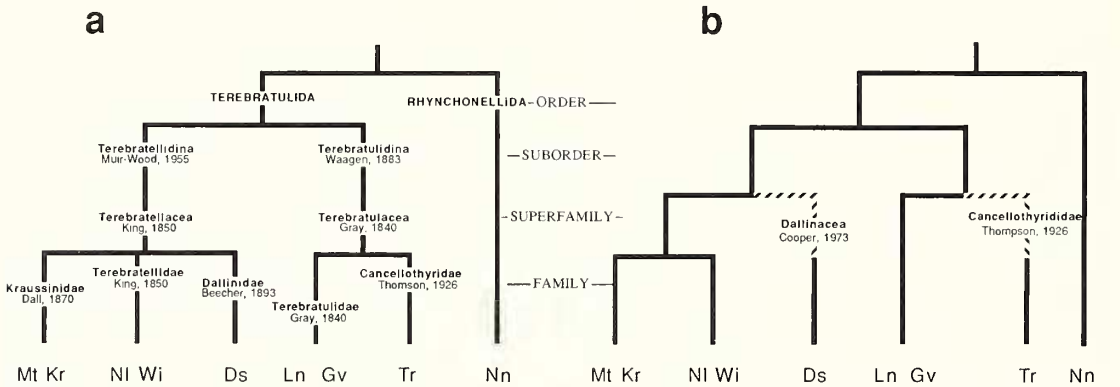
study) there is excellent congruence between genetic (Cohen *et al.* 1991) and both immunological (Collins *et al.* 1988) and morphological (Cohen *et al.* 1991) data.

The immunological approach is particularly useful for groups such as the brachiopods, where live sampling is only rarely possible. By isolating protected macromolecules (intra-crystalline skeletal glycoproteins), sampling of spirit-stored and dried museum specimens was possible, greatly extending the range of available material. More significantly, *fossil* organic matter isolated from Pleistocene and Pliocene skeletons could also be screened immunologically (Collins *et al.* in press). If it is possible, using immunological distances, to establish a phenogram for Recent brachiopods, this could then be used as a benchmark against which to compare the pattern of reactions of fossil extracts (when tested with the same range of sera), to assess the quality of preserved molecular information (Collins *et al.* in press).



TEXT-FIG. 1. *a*, systematic sub-division of the Terebratulida based on the Treatise (Williams *et al.* 1965). *b*, modifications of the Terebratulida by the inclusion of two new post-Treatise superfamilies proposed by Cooper (1973, 1981).

A total of nine brachiopod antisera were prepared against skeletal glycoproteins. Although the taxonomic distribution of the genera investigated has been dictated primarily by the availability of sufficient quantities of material for antiserum preparation, it has been possible to include taxa which represent all major groups of living articulate brachiopods. Not surprisingly, the number of genera available from each group closely reflects their relative abundance in Recent brachiopod faunas. Thus, five genera were available from among the long-looped terebratulids (which dominate present-day faunas), four of which were assigned to two families of the Superfamily Terebratellacea, while the remaining genus was classified within the Superfamily Dallinacea (Text-fig. 1*b*). Three short-looped terebratulids were available, representing two discrete families which, depending upon interpretation, represented either one (Text-fig. 1*a*) or two (Text-fig. 1*b*) discrete superfamilies. The rhynchonellid genus *Notosaria* was a convenient outgroup for the investigation of terebratulid phylogeny. Roughly 10% of all living brachiopod genera are therefore included in this first comprehensive investigation of the biochemical systematics of brachiopods.

MATERIALS AND METHODS

Species and sources used in the immunological investigation are listed in Table 1. Shells were soaked in concentrated NaOCl (12% active chlorine) solution overnight prior to further handling to remove surface contaminants. Additional NaOCl was added for a minimum 48 hours to remove the inter-crystalline organic matrix, and thereby weaken the shell, liberating the fibres of the secondary shell layer (Collins 1986). It was necessary to treat the rhynchonellid *Notosaria* for a week before shell softening was complete. The fibres were isolated from the remainder of the shell material as previously described (Collins *et al.* 1988) and exhaustively rinsed in double-distilled water prior to freeze drying.

TABLE 1. Species, with localities, used in the immunological distance study.

Taxon	Locality	Prep.	Abbrev.	Serum
Terebratellidae				
Waltonia (*Terebratella*) *inconspicua* (Sowerby)	Christchurch, N. Zealand	Fibre	Wi	K5040
Neothyris lenticularis (Deshayes)	Foveaux Strait, N. Zealand	Protein	NL	427
Dallinidae				
Dallina septigera (Lovén)	Hebridian Rise, Scotland	Fibre	Ds	K5007
Kraussinidae				
Kraussina rubra (Pallas)	Southern Tip, S. Africa	Fibre	Kr	801
Megerlia truncata (Gemlin)	Corsica, Mediterranean	Fibre	Mt	K5053
Cancellothyridae				
Terebratulina retusa (L.)	Firth of Lorn, Scotland	Powder	Tr	K4962
Terebratulidae				
Liothyrella neozelandica (Thomson)	Foveaux Strait, N. Zealand	Fibre	Ln	802
Gryphus vitreus (Born)	Corsica, Mediterranean	Powder	Gv	803
Rhynchonellida				
Notosaria nigricans (Sowerby)	Christchurch, New Zealand	Fibre	Nn	K5038

Intra-crystalline macromolecules were isolated from the secondary layer fibres by de-mineralization in 20% wt/vol disodium ethylene diamine tetraacetic acid (EDTA, pH 8), and the EDTA was subsequently removed by ultra-filtration across an Amicon YM 5 or 10 filter (5 or 10 kD). This treatment yielded approximately 6 mg of water-soluble organic matter per 20 g of fibre preparation.

Special treatments were applied to three samples. For *Gryphus vitreus* it was necessary to use shell powders since secondary layer fibres could not be isolated (this genus develops a tertiary shell layer), *Megerlia truncata*, for which little material was available, was dialyzed against EDTA using the technique of Weiner and Lowenstam (1980), and for *Neothyris lenticularis* a single protein (45 kD) cut from a sodium dodecyl sulphate polyacrylamide gel was used for immunization.

Antisera were prepared using 500 μg aliquots of sample immunized subcutaneously into New Zealand white rabbits according to the following schedule. Primary immunization with Freund's complete adjuvant, followed by three secondary injections with incomplete adjuvant at two to three week intervals, with bleeds after the second and third booster immunizations. Antisera were stored with 0·002% NaN_3 in aliquots at -20 °C.

The IgG fraction of each antiserum was prepared using a crude ammonium sulphate precipitation technique. Saturated $(NH_4)_2SO_4$ was added drop by drop to serum on ice to final volume of 50%. The serum was left for 30 minutes at 4 °C and then centrifuged at 14000 rpm for 10 minutes. The supernatant was carefully pipetted off and the pellet re-suspended in Tris buffered saline (20 mM Tris, 0·9% wt/vol NaCl, pH 7·5; TBS).

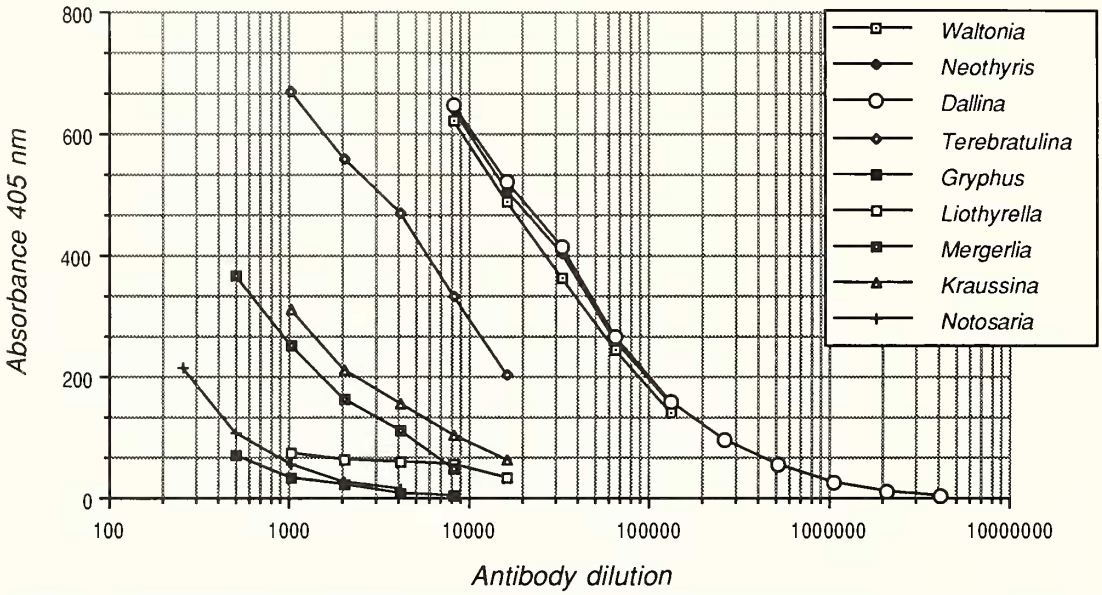
Crude antigen preparations for the immunological assay were produced by dissolving a slight excess of shell fibre (or powder) preparation in 20% wt/vol EDTA (a ratio of 0·046 g fibre/1 ml EDTA), and then centrifuging to remove the residual undissolved carbonate.

An enzyme-linked immunosorbent assay (ELISA; Harlow and Lane 1988) was used to measure immunological distances among taxa. Both antigen (crude EDTA extract) and homologous antiserum were used for all taxa examined, and all antigen-antibody combinations were produced. 10 μl of each crude antigen solution, diluted to 100 μl in 20% wt/vol EDTA, was pipetted onto the microtitre plate which was then incubated at 37 °C for 90 minutes. Unbound antigen was washed away by rinsing three times with washing buffer (TBS to which had been added 0·05% wt/vol of the ionic detergent Tween 20). The wells were blocked for 30 minutes with 2% wt/vol gelatin diluted in TBS. Antisera diluted in 0·2% wt/vol gelatin/TBS/Tween 20 were added to given wells and incubated overnight at 4 °C for 1·5 hours at 37 °C, following which any unbound material was washed away. Alkaline phosphatase labelled goat-anti-rabbit-affinity purified IgG (GAR-AP, Sigma) diluted 1:5000 in gelatin/TBS/Tween 20 was added to the wells for 1 hour at 37 °C after which the wells were rinsed five times with TBS/Tween, once with TBS and then allowed to stand for 2 minutes with phosphatase substrate buffer (pH 9·2), prior to the addition of disodium p-nitrophenyl phosphate (Sigma). After 15 minutes the reaction was stopped by the addition of 50 μl of 1 M NaOH, and the absorbence

at 405 nm of the wells read immediately with a Titretek Multiskan Plus, automated plate reader. The extent of colour development is proportional to the amount of first antibody bound.

Immunological distance was calculated using the formula ID = 100 × log10 (100/Δ), where Δ is the mean reciprocal % cross-reactivity (homologous antigen reactivity = 100%; see Table 2). These distances were obtained from the linear regions of semi-logarithmic binding curves plotted using a series of antibody concentrations for each combination of antigen and antibody (Text-fig. 2). Reciprocal distances were averaged for each combination and means of duplicates were used for clustering.

Tree diagrams were constructed either using the method of UPGMA (Sneath and Sokal 1973) or Fitch and Margoliash (1967), as represented by FITCH in the program package PHYLIP, written by Joseph Felsenstein (University of Washington, Seattle).

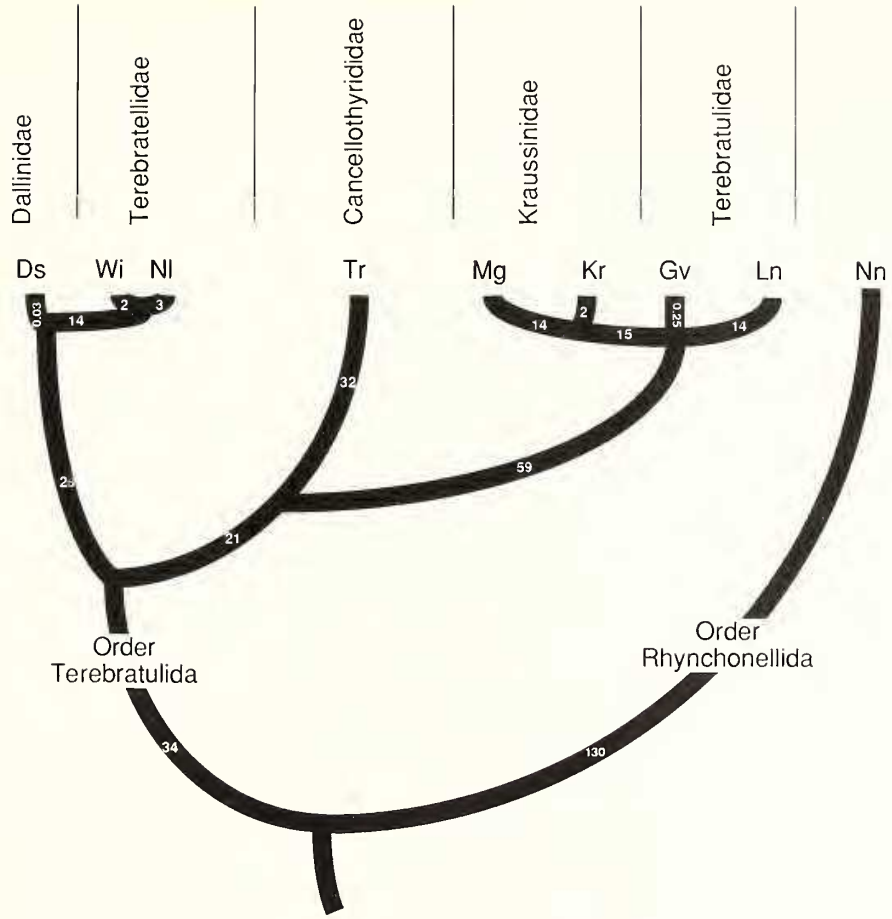


TEXT-FIG. 2. Immunological binding curves. A series of dilutions of the antiserum raised against *Dallina*, tested against all nine antigens. Immunological distance (ID) between species is 100 times the log of the antiserum dilution required to give the same binding for two different species. ID can be determined directly from the linear portions of the binding curves.

RESULTS AND DISCUSSION

Taxonomic framework

In the brachiopod Treatise, the Order Terebratulida was divided into two superfamilies, the long-looped Terebratellacea and the short-looped Terebratulacea. A simplified graphical representation of the Treatise classification down to family level is shown in Text-figure 1*a*. The classification is based entirely on comparative morphology, and the graphical representation should not be confused with the phenograms used to portray the immunological data. The 'clustering' unit (i.e. vertical axis) is taxonomic hierarchy for the representation of the Treatise classifications, while for the immunological data it is a numerical scale reflecting the degrees of reactivity of the various antisera. To emphasize this point, the analyses of immunological data are shown with the most closely related taxa at the top (i.e. the immunological distance between clusters increases downwards; Text-fig. 3), while the morphological interpretation is shown in the reverse orientation with the lower levels of the taxonomic hierarchy (i.e. families, genera) at the bottom and orders, etc. at the top (Text-fig. 1).



TEXT-FIG. 3. UPGMA phenogram of the immunological distances of nine genera of articulate brachiopods (data from Table 2).

There have been a number of suggested modifications to the Treatise classification some of which have been incorporated into Text-figure 1*b*. The major complication with such modified schemes is that they only deal with relatively few genera, and may leave many unanswered questions about the authors' intended taxonomic assignments, if any, for taxa not discussed. The problem can be circumvented in this paper by including only those groups for which immunological data are currently available. This involves two new superfamilies which have been proposed by Cooper (1973, 1981) and which, in effect, subdivides the two terebratulid superfamilies listed in the Treatise.

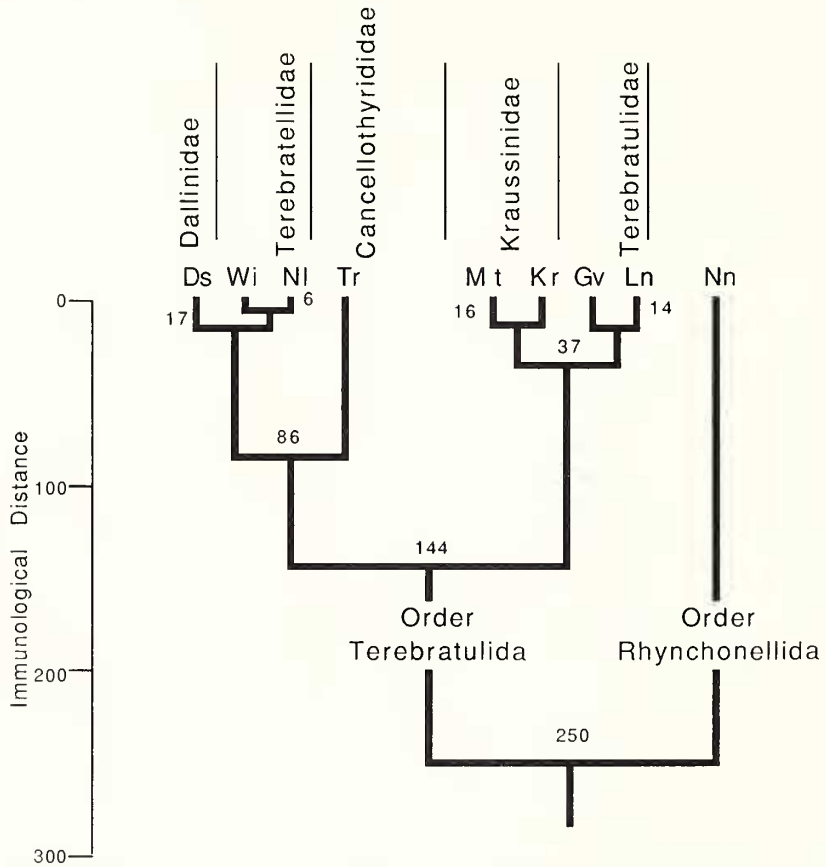
Immunological distances

Immunological distances among genera of the order Terebratulida are presented in Table 2 and the UPGMA and Fitch-Margoliash dendrograms based on this data set are given in Text-figures 3 and 4.

At one extreme, the outgroup rhynchonellid *Notosaria nigricans* was the least reactive with all eight terebratulid antisera, and hence was well separated from the terebratulids in both phenograms (Text-figs 3 and 4). At the other extreme, all confamilial genera clustered together in both forms of analysis (e.g. *Megerlia* with *Kraussina*, *Gryphus* with *Liothyrella*, and *Neothyris* with *Waltonia*;

TABLE 2. Immunological distances among the articulate brachiopods from ELISA of intra-crystalline macromolecules. Distances represent means of reciprocal distances obtained from binding curves. See Table 1 for species abbreviations.

	Wi	Ds	NL	Tr	Gv	Li	Mt	Kr	Nn
Waltonia inconspicua	0	15	6	100	169	177	204	117	301
Dallina septigera		0	18	77	118	129	154	170	168
Neothyris lenticularis			0	88	156	149	130	132	239
Terebratulina retusa				0	134	70	146	206	253
Gryphus vitreus					0	14	45	15	193
Liothyrella neozelandica						0	34	54	272
Megerlia truncata							0	16	284
Kraussina rubra								0	306
Notosaria nigricans									0



TEXT-FIG. 4. Fitch-Margoliash phenogram of the data from Table 2. Note that while the overall topology is similar to Text-figure 3, the cancellothyrid lineage (*Terebratulina*) arises from the terebratulid, and not the TLD, lineage.

Text-figs 3 and 4). These results, at the highest and lowest level of the taxonomic hierarchy investigated, are entirely consistent with established morphology-based brachiopod systematics, and reinforce our contention that intra-skeletal macromolecules are an important source of phylogenetic information (Collins *et al.* 1988).

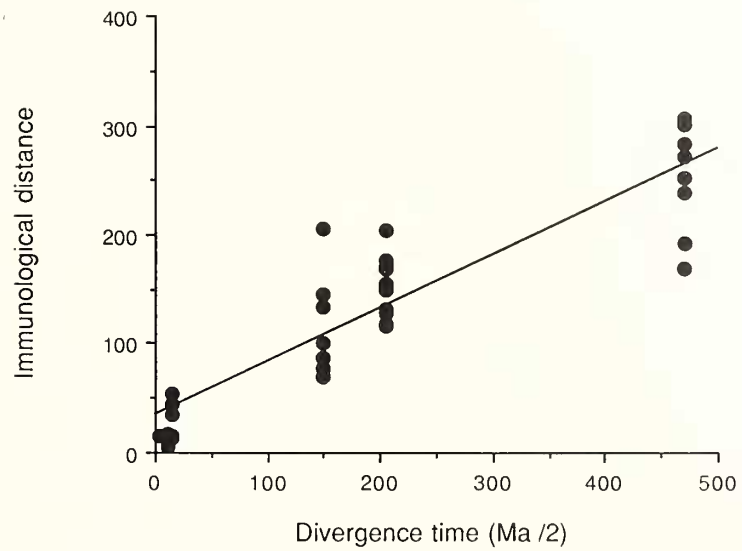
Within the terebratulids, the immunological distances distinguish three main clusters (Text-figs 3 and 4). The novel aspect of these three main clusters is that they do not coincide with the long- and short-looped stocks which, as mentioned above, currently represent the primary subdivisions of the terebratulids. Instead, two represent respectively the Cancellothyrididacea (raised to superfamily status by Cooper 1973) and a sub-group of the Terebratellacea (the so-called TLD group of Collins *et al.* 1988). The third is more heterogeneous, including both a long-looped family (the Kraussinidae) and short-looped superfamily Terebratulacea.

The fact that the three-way clustering pattern is confirmed by two different methods of analysing the data (Text-figs 3 and 4), and is based on fully reciprocal immunological distances, confirms that this is an accurate reflection of the phylogenetic relationships among living terebratulids.

Plotting the immunological distances of the major nodes against estimated divergence times (Table 3) reveals a strong correlation (Text-fig. 5). However, given that there is no universal 'molecular evolutionary clock' (e.g. Britten 1986), it is necessary to put this apparent correlation into context.

TABLE 3. Estimated divergence time of major branches used in regression of ID against range.

Divergence	mean ID	Time (Ma)	Rationale (first occurrence of...)
No divergence	0	0	
Wi from NL	6	12	genus *Waltonia*
Mt from Kr	16	3	genus *Kraussina*
Li from Gv	14	15	genus *Liothyrella*
DI from Wi, NL	16	15	subfamily Neothyridinae
Mt, Kr from Li, Gv	37	15	family Kraussinidae
Tr from DI, Wi, NL	100	150	genus *Terebratulina*
Mt, Kr, Li, Gv from DL, Wi, NL	163	205	subfamily Terebratulinae
Nn from others	295	470	Order Spiriferida (ancestral to the Terebratulida)



TEXT-FIG. 5. Plot of immunological distance against range. Divergence times are estimated from the ages given in Table 3.

The divergence times (listed in Table 3) used for the analysis are based on the first appearance of the most recent probable ancestor, but these are not always well defined, and are subject to significant change as continuing investigation of fossil brachiopods extends or reduces the known range of taxa. In addition, although the approach of using polyclonal antisera against a large number of different determinants will generate an averaged clock speed, which is beneficial, the structure of individual antigenic determinants is not known. The immunological determinants of the glycoproteins are believed to be mainly sugar moieties (due to their sensitivity to periodate and their insensitivity to proteinase K treatments; Collins *et al.* in press), the production of which is controlled by complex pathways, of whose evolutionary patterns we remain ignorant.

Evolution of the Order Terebratulida

The Order Terebratulida, which is first recorded from the Lower Devonian, is characterized by the possession of a distinctive internal skeleton, 'the loop'. The traditional interpretation of evolution within this order is of considerable 'experimentation' with the internal skeleton in the early Devonian. The period of diversification was relatively short (e.g. Stehli 1965; Rudwick 1970; Williams and Hurst 1977), with two distinct lineages diverging in the late Devonian, giving rise to the present-day Terebratulacea (short loop) and Terebratellacea (long loop).

The serotaxonomic data clearly indicate that this interpretation is in considerable need of revision. Assuming the minimum age for diversification of the rhynchonellid and terebratulid lineages is approximately 470 Ma at the first appearance of the order Spiriferida (believed to be ancestral to the Terebratulida), then the great immunological distances between this primary division and subsequent diversification suggests that surviving lineages diversified considerably later than the Devonian.

The immunological data would suggest that the three modern-day lineages identified in this study (TLD terebratellaceans, the cancellothyrids, and the 'terebratulaceans', the latter including the Kraussinidae) diverged from each other at around the same time (Text-figs 3 and 4). An early Mesozoic diversification is implied by the immunological data, which is consistent with the appearance of the first terebratellacean and terebratulacean genera in the Upper Triassic, and the earliest cancellothyrids from the Middle to Upper Jurassic (Muir-Wood *et al.* 1965).

The three major lineages have subsequently undergone different patterns of evolution. The most morphologically conservative have been the cancellothyrids, which have changed very little since the late Jurassic, and which consequently have the largest and older surviving terebratulid genus *Terebratulina*, surely a case for revision?

The small immunological distances between the three members of the TLD cluster imply a high degree of relatedness, which runs contrary to the assumption that members of this lineage diversified as early as the Upper Cretaceous (Muir-Wood *et al.* 1965). Conversely, the immunological distances which separate the Kraussinidae from the Terebratulidae are greater than is anticipated from a geological record for the former family which extends back only into the Miocene.

The clustering of *Macandrevia*, previously included in the Dallinidae (Muir-Wood *et al.* 1965), or Laqueidae (Richardson 1975), within the 'kraussinid' group (Text-figs 3 and 4) illustrates the limited value of loop morphology for familial assignment. The value of the loop as a taxonomic character is further reduced when (as is common in fossil samples) its ontogeny is difficult to decipher. The serological data suggest a much closer relationship between *Dallina* and the Terebratellidae than a Cretaceous diversification would imply. It is clear that future studies will have to determine whether the accepted familial assignments of Cretaceous genera are justified, with their added implication that both the laqueid and terebratellid lineages (*sensu* Richardson 1975) can be traced back to the Cretaceous.

The phylogeny of the Kraussinudae has recently been discussed by Brunton and Hiller (1990). If the lineage also includes the Megathyrididae, as suggested by our previous study (Collins *et al.* 1988), and expected from current classification, the divergence of this lineage would be placed in the late Mesozoic, which more closely corresponds with the immunological distances observed. The major revelation arising from the immunological data, although anticipated in our earlier study

(Collins *et al.* 1988), is not the divergence time of the kraussinid cluster, but its origin. The immunological data clearly indicate that the lineage is derived, not from the long-looped terebratellaceans (TLD group), but from the short-looped (terebratulacean) stock. In reviewing the phylogeny of the kraussinids, Brunton and Hiller (1990) justifiably comment that our preliminary results (Collins *et al.* 1988) linking the kraussinids to the terebratulaceans were surprising, given that there is no obvious kraussinid-like ancestor within this lineage. However, additional antisera raised for this investigation, against *Kraussina*, *Megerlia*, *Liothyrella*, and *Gryphus*, all confirm the original finding. This result has major taxonomic implications, not only for the classification of the kraussinids, but for the significance of the loop as a subordinal taxonomic character.

Taxonomic Implications

Immunological data cannot on their own determine the taxonomic relationships of terebratulid brachiopods, because only Recent taxa are involved in the immunological distance experiments. The data only relate to the phylogenetic relationships of the families to which the included genera are assigned, and these assignments may be incomplete or wrong (e.g. *Macandrevia*; Collins *et al.* 1988). However, the immunological study highlights relationships which contradict current morphological classifications.

The simplified cardinalia and distinctive loop morphology of the cancellothyrids make this the most homogeneous of the extant terebratulid lineages.

The TLD terebratellaceans are characterized by supporting hinge plates on the median septum, which distinguishes these terebratellaceans from the 'aberrant' genus *Macandrevia*, but this latter organization is also seen in some Palaeozoic terebratulids and in other articulate brachiopod orders. The loop, which is best developed within this group, has previously been cited as the major discriminatory feature in the terebratulids (e.g. Stehli 1965). However, ontogenetically, there is a strong relationship between long- and short-looped forms. The earliest stages in the development of the descending branches of the calcareous loop up to their fusion with the median septum are the same for all three extant lineages. Elliott (1953, 1957) notes that the first-formed calcareous support for all modern long-looped brachiopods (with the exception of *Argyrotheca*) always includes a dorsal median septum. Of the Palaeozoic superfamilies of terebratulids believed to be ancestral to modern forms, all contain genera which possess median septa, but there is no clear evidence for the involvement of the septum in the ontogeny of the loop in either the Stringocephalacea or Zeilleriacea. If in these two superfamilies a long loop was derived simply by anterolateral growth of a short dielasmatacean-like loop, then the role of the median septum in all modern long-looped lineages is significant. The fixing of *one* novel character, a link between an acceleration in the time and rate of growth of the median septum coincident with that of the descending branches was probably all that was necessary to pave the way for the Mesozoic and Cenozoic diversifications (Text-fig. 6).

Immunological distances provide an important new perspective on terebratulid evolution, and pose problems for current morphologically-based taxonomy. Morphological taxonomy of a group with such a 'simple' skeleton is prone to error, yet molecular taxonomy is generally inapplicable to a predominantly fossil group. Therefore, a major taxonomic revision of this order appears inevitable, and it should proceed as a rigorous re-analysis of skeletal ontogeny and morphology in the light of a more widespread molecular investigation.

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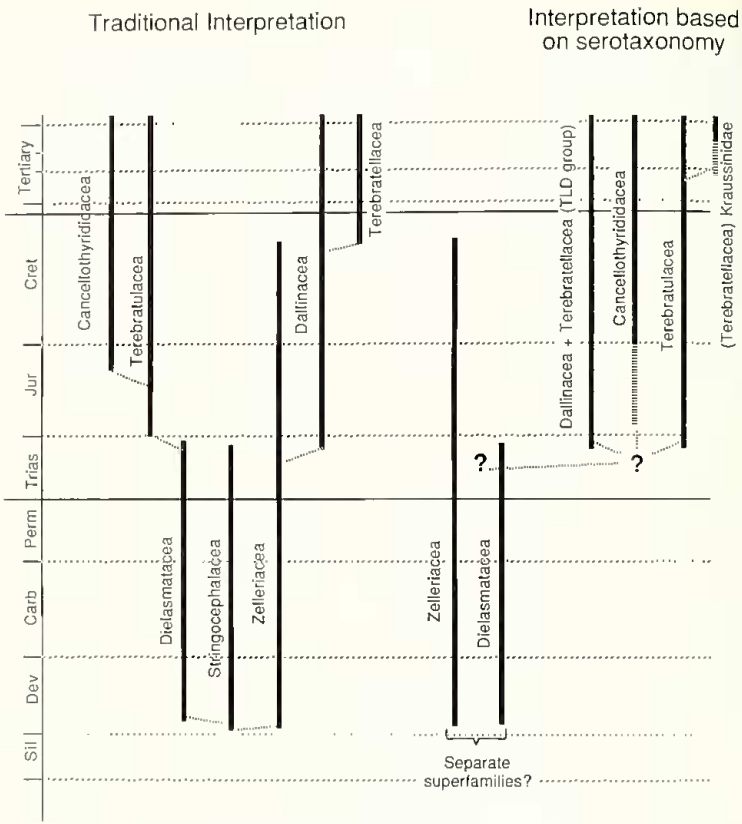

TEXT-FIG. 6. Terebratulid phylogeny (after Williams and Rowell 1965; Williams and Hurst 1977) reinterpreted in the light of the serotaxonomic data.

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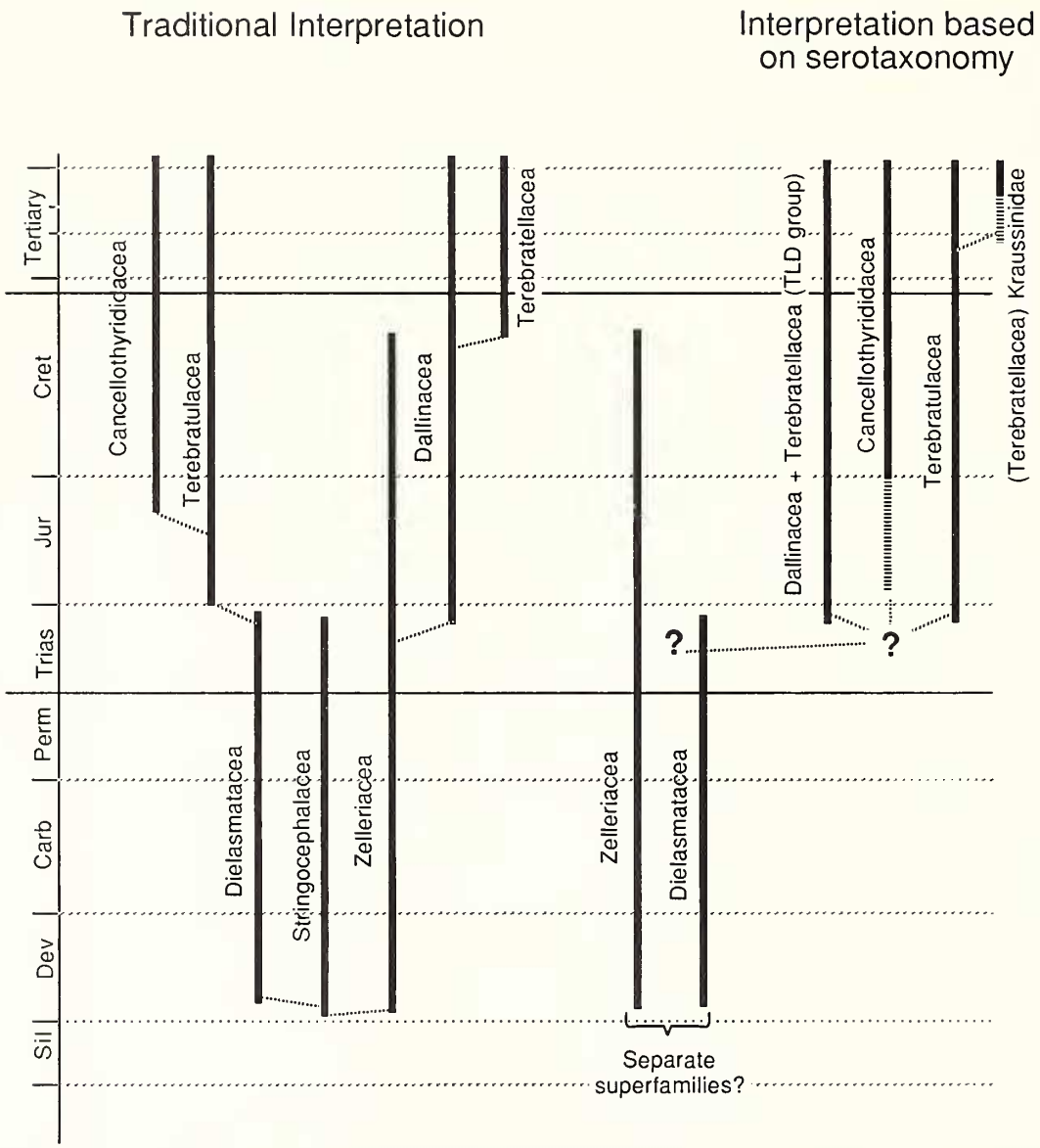

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